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Two Novel Self-compatible *S* Haplotypes in Peach (*Prunus persica*)

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Peach (*Prunus persica*) as a species is self-compatible (SC), although most other *Prunus* fruit tree species are partially or fully self-incompatible. We previously identified 3 mutated *S* haplotypes, S^1, S^2, and S^{2m}, that confer self-compatibility on commercial peach cultivars for fruit production. In this report, we identified 2 novel SC *S* haplotypes, S^3 and S^4, among 130 peach cultivars and strains consisting mainly of ornamental cultivars and wild strains. The S^3 haplotype was found only in ornamental cultivars, while the S^4 haplotype was found mainly in wild strains. *S-RNase*s in the S^3 and S^4 haplotypes appeared to have no defects in their primary structures. *S haplotype-specific F-box* (*SFB*) sequences were also present in the *S* locus downstream of the S^3- and S^4-*RNase*s. These *SFB* sequences were in a reverse transcriptional orientation as has been reported in most other functional *Prunus S* haplotypes; however, both SFB^3 and SFB^4 appeared to be mutated. DNA sequencing of the entire downstream region of SFB^3, extending about 12 kbp to the stop codon of *S-RNase*, revealed the presence of a premature stop codon 975 bp downstream from the SFB^3 start codon. No sequence homologous to *SFB* downstream of the stop codon was found. There was a 4946 bp insertion in the middle of SFB^4. The original SFB^4 sequence, obtained by removing the inserted sequence, encodes a typical SFB. Based on the 3 previously identified peach *S* haplotypes, we supposed that the S^3 and S^4 haplotypes were also SC pollen part mutant (PPM) *S* haplotypes. Here, we also discuss possible reasons for all peach *S* haplotypes identified so far having the PPM SC *S* haplotype.

Key Words: F-box protein, pollen part mutation, self-incompatibility, SFB, S-RNase.

Introduction

Self-incompatibility is a genetically controlled pollen-pistil recognition mechanism that prevents self-fertilization and promotes outcrossing (de Nettancourt, 2001). Most *Prunus* (family Rosaceae) fruit tree species exhibit a homomorphic gametophytic self-incompatibility (GSI) system in which self/nonself-recognition is controlled by a single multiallelic locus, called the *S* locus (Tao and Iezzoni, 2010; Yamane and Tao, 2009). A self-incompatibility reaction is triggered when the same *S*-allele specificity is expressed in both the pollen and pistil. Thus, growth of a pollen tube bearing either of the 2 *S*-allele specificities carried by the recipient pistil is arrested in the style. During the last 2 decades, the molecules involved in GSI recognition have been identified in several plant species. It is now known that 2 separate genes, the S-ribonuclease gene (*S-RNase*) and *S* haplotype-specific F-box protein gene (*SFB*) at the *S* locus, control male and female specificities, respectively, in *Prunus* (Ushjima et al., 2003; Yamane et al., 2003). The term "*S* haplotype" is used to describe variants of the *S* locus, whereas the term "*S* allele" is used to

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describe the variant of a given *S* locus gene.

Mutations in *S-RNase* that lead to dysfunction of the S-RNase enzyme are known to confer self-compatibility commonly in rosaceous and solanaceous plants that have the S-RNase-based GSI system. In sour cherry (*P. cerasus*) (Yamane et al., 2001), Japanese plum (*P. salicina*) (Watari et al., 2007), and almond (*P. dulcis*) (Hanada et al., 2009), self-compatibility is conferred by a low level of *S-RNase* transcription that leads to a low level of S-RNase accumulation in the style. A frameshift or substitution mutation in *S-RNase* that led to the translation of a dysfunctional S-RNase was also reported to confer self-compatibility in sour cherry (Tsukamoto et al., 2008, 2010). Mutations in the pollen *S* gene, however, resulted in different outcomes depending on the taxon or the family that showed the S-RNase-based GSI. Although mutations that disrupt the pollen *S* determinant F-box gene in Solanaceae and Plantaginaceae are supposed not to confer self-compatibility, these mutations did result in self-compatibility in *Prunus* (Sonneveld et al., 2005; Tao and Iezzoni, 2010; Ushijima et al., 2004; Yamane and Tao, 2009). Taken together, these findings confirm that a mutation in either *S-RNase* or *SFB* confers self-compatibility in *Prunus* (Tao and Iezzoni, 2010; Yamane and Tao, 2009).

Peach (*Prunus persica*) as a species is self-compatible (SC), although most other fruit tree species in the genus *Prunus* are partially or fully self-incompatible. We previously investigated the *S* locus of 40 peach cultivars and strains consisting mainly of Japanese commercial cultivars for fruit production (Tao et al., 2007). Among them, we identified 3 *S* haplotypes, S^1, S^2, and S^{2m}, all of which appeared to encode mutated dysfunctional SFB (Tao et al., 2007). The S^1 haplotype is a pollen part mutant (PPM) version of the almond S^k haplotype, while the S^2 haplotype is a PPM version of the Japanese plum S^a haplotype. The S^{2m} haplotype is a mutant version of the peach S^2 haplotype, in which both *S-RNase* and *SFB* are mutated, while only *SFB* is mutated in the S^2 haplotype. Considering that most Japanese commercial peach cultivars for fruit production are descendants of 'Shanhai Suimitsuto (Shang Hai Shui Mi Tao)', a Chinese cultivar known as 'Chinese Cling' (Yamamoto et al., 2003), there should be unidentified novel peach SC *S* haplotypes in cultivars and wild strains that originated from other regions.

In this study, we identified 2 novel SC *S* haplotypes, S^3 and S^4, among 130 peach cultivars and strains consisting mainly of ornamental cultivars and wild strains. The *S-RNases* in the S^3 and S^4 haplotypes appeared to be intact, while the *SFB*s in both *S* haplotypes were truncated. As reported previously for the 3 identified peach *S* haplotypes, the S^3 and S^4 haplotypes were assumed to be PPM SC *S* haplotypes. Here, we discuss the possible reasons why all peach *S* haplotypes identified so far are PPM SC *S* haplotype.

Materials and Methods

Plant materials

A total of 130 peach cultivars and strains consisting mainly of ornamental cultivars and wild strains were selected from peach germplasm collections at the University of California at Davis (USA), the NARO Institute of Fruit Tree Science (Japan), the Research Institute for Agriculture Okayama Prefectural Technology Center for Agriculture, Forestry and Fisheries (Japan), and the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón (Spain). The origin and description of all cultivars analyzed are shown in Table 1. In addition to the 130 cultivars and strains, 2 Japanese fresh fruit cultivars, 'Shimizuhakuto' (S^1S^{2m}) and 'Chiyomaru' (S^2S^2), grown at the experimental farm of Kyoto University, were used as references for the *S* haplotypes in this study. Young leaves were collected in the spring of 2005–2007, frozen in liquid nitrogen, lyophilized, and stored at –20°C until used.

DNA extraction

Total DNA was isolated from lyophilized young leaves using the CTAB method or the Nucleon Phytopure plant and fugal DNA extraction kit (GE Healthcare, Piscataway, NJ, USA) as described previously (Hanada et al., 2009).

PCR-based genotyping

Total isolated DNA was used as a template for PCRs using the Pru-C2 and Pru-C4R primer set as described previously (Tao et al., 1999). This primer set was designed to detect the length polymorphism in the second intron in *S-RNase*. Because it appeared that PCRs using the Pru-C2/Pru-C4R primer set were unable to amplify S^4*-RNase* effectively, we occasionally performed S^4*-RNase* allele-specific PCRs using the S4-RNase F3 and S4-RNase R5 primer set to determine the presence of the S^4*-RNase* allele when it was present heterozygously with other *S-RNase* alleles. A primer set for the dCAPS marker, S2Dra-F and S2Dra-R, was used to distinguish between S^2*-RNase* and S^{2m}*-RNase*, as described by Tao et al. (2007). The oligonucleotide primer sequences used in this study are listed in Table 2.

DNA gel blot analysis

Five micrograms of total DNA was digested using *Eco*RI or *Hin*dIII, run on 0.8% agarose gel, and transferred to a nylon membrane (Biodyne Plus; Pall, Port Washington, NY, USA). Hybridization was performed using a DIG-dUTP-labeled probe (Roche Diagnostics, Basel, Switzerland) obtained by PCR labeling with sweet cherry S^1*-RNase* cDNA and the Pru-C2/Pru-C4R primer set, and washed under low stringency conditions, as described previously (Tao et al., 1999). Hybridization signals were detected using chemiluminescent substrate CDP-Star (New England Biolabs, Ipswich, MA, USA)

Table 1. Cultivars and strains used in this study and their *S* haplotypes.

No.	Cultivar or strain	*S* haplotype[z]	Planting location[y]	Origin
1	Nepal Peach Col. No. 84-102	S^1S^1	NIFTS	Nepal
2	Nepal Peach Col. No. 84-114	S^1S^1	NIFTS	Nepal
3	Nepal Peach Col. No. 84-120	S^1S^1	NIFTS	Nepal
4	Nepal Peach Col. No. 84-125	S^1S^1	NIFTS	Nepal
5	Nepal Peach Col. No. 84-131	S^1S^1	NIFTS	Nepal
6	Nepal Peach Col. No. 84-133	S^1S^1	NIFTS	Nepal
7	Nepal Peach Col. No. 84-137	S^1S^1	NIFTS	Nepal
8	Nepal Peach Col. No. 84-155	S^1S^1	NIFTS	Nepal
9	Nepal Peach Col. No. 84-B-201	S^1S^1	NIFTS	Nepal
10	Nepal Peach Col. No. 84-B-206	S^1S^1	NIFTS	Nepal
11	Nepal Peach Col. No. 85-119-B	S^1S^1	NIFTS	Nepal
12	Nepal Peach Col. No. 85-125	S^1S^1	NIFTS	Nepal
13	Nepal Peach Col. No. 85-379	S^1S^1	NIFTS	Nepal
14	Nepal Peach Col. No. 85-4021	S^1S^1	NIFTS	Nepal
15	Nepal Peach Col. No. 85-4022	S^1S^1	NIFTS	Nepal
16	Nepal Peach Col. No. 85-4067	S^1S^1	NIFTS	Nepal
17	Nepal Peach Col. No. 85-4083	S^1S^1	NIFTS	Nepal
18	Nepal Peach Col. No. 85-4087	S^1S^1	NIFTS	Nepal
19	Nepal Peach Col. No. 85-4092	S^1S^1	NIFTS	Nepal
20	Nepal Peach Col. No. 86-IV-36	S^1S^1	NIFTS	Nepal
21	Pakistan Prunus Col. No. 95-26	S^1S^1	NIFTS	Pakistan
22	Pakistan Prunus Col. No. 95-27	S^1S^1	NIFTS	Pakistan
23	1470.9 B	S^1S^1	UC Davis	Pakistan
24	1474.10 B	S^1S^1	UC Davis	Pakistan
25	1475.10 C	S^1S^1	UC Davis	Pakistan
26	1477.10 B	S^1S^1	UC Davis	Pakistan
27	Churkoc	S^1S^1	UC Davis	Pakistan
28	Hunshu	S^1S^1	UC Davis	Pakistan
29	Thulu	S^1S^1	UC Davis	Pakistan
30	Hekito (Double colored)	S^1S^1	Okayama	China (Ornamental Peach)
31	Okayama Yaseitou Asahikawa-2	S^1S^1	NIFTS	Japan (Wild Peach)
32	Okayama Yaseitou Kamogawa-1	S^1S^1	NIFTS	Japan (Wild Peach)
33	Nagano Yaseitou-Wase	S^1S^1	NIFTS	Japan (Wild Peach)
34	Noto 3	S^1S^1	NIFTS	Japan (Wild Peach)
35	Terute Suimitsu	S^1S^1	NIFTS	Japan (Ornamental Peach)
36	Nepal Peach Col. No. 84-115	S^1S^2	NIFTS	Nepal
37	Nepal Peach Col. No. 84-119	S^1S^2	NIFTS	Nepal
38	Chalpachu	S^1S^2	UC Davis	Pakistan
39	Noto 2	S^1S^2	NIFTS	Japan (Wild Peach)
40	Noto 8	S^1S^2	NIFTS	Japan (Wild Peach)
41	Jing Hong	S^1S^{2m}	NIFTS	China
42	Jing Hong Tao	S^1S^{2m}	NIFTS	China
43	Shen Zhou Bai Xue	S^1S^{2m}	NIFTS	China
44	Hoko	S^1S^{2m}	Okayama	China
45	Tououbo	S^1S^{2m}	Okayama	China
46	Hekito (Beni)	S^1S^{2m}	Okayama	China (Ornamental Peach)
47	Kimumu Nakamineyuumei	S^1S^{2m}	NIFTS	Japan (Wild Peach)
48	Yaezaki Bantou O.P. No. 1	S^1S^3	NIFTS	Japan (Ornamental Peach)
49	Okayama Yaseitou Asahikawa-1	S^1S^4	NIFTS	Japan (Wild Peach)
50	Nepal Peach Col. No. 84-121	S^1S^4	NIFTS	Nepal
51	Okayama Yaseitou Kamogawa-2	S^1S^4	NIFTS	Japan (Wild Peach)
52	Nagano Yaseitou-Bansei	S^1S^4	NIFTS	Japan (Wild Peach)
53	Akahayazaki	S^2S^2	NIFTS	Japan (Ornamental Peach)
54	Akashidare	S^2S^2	NIFTS	Japan (Ornamental Peach)
55	Amami Yaseitou-1	S^2S^2	NIFTS	Japan (Wild Peach)
56	Amami Yaseitou-2	S^2S^2	NIFTS	Japan (Wild Peach)
57	Chichibu 1	S^2S^2	NIFTS	Japan (Wild Peach)
58	Chichibu 4	S^2S^2	NIFTS	Japan (Wild Peach)
59	Nepal Peach Col. No. 84-522	S^2S^2	NIFTS	Nepal
60	Nepal Peach Col. No. 86-III-210	S^2S^2	NIFTS	Nepal
61	Nepal Peach Col. No. 86-V-169	S^2S^2	NIFTS	Nepal
62	Nepal Peach Col. No. 87-VIII-67	S^2S^2	NIFTS	Nepal
63	Pakistan Prunus Col. No. 95-25	S^2S^2	NIFTS	Pakistan
64	Golden Glory	S^2S^2	NIFTS	United States
65	Golden Prolific	S^2S^2	NIFTS	United States
66	Silver Prolific	S^2S^2	NIFTS	United States
67	Swatow	S^2S^2	NIFTS	China (Ornamental Peach)
68	Juseitou-Aka-Yae	S^2S^2	NIFTS	Japan (Ornamental Peach)
69	Juseitou-Pink-Yae	S^2S^2	NIFTS	Japan (Ornamental Peach)

Table 1. Continued

No.	Cultivar or strain	*S* haplotype[z]	Planting location[y]	Origin
70	Da Tao	S^2S^2	NIFTS	China
71	Kemomo Nagoshijou	S^2S^2	NIFTS	Japan (Wild Peach)
72	Ku Tao 1	S^2S^2	NIFTS	Taiwan
73	Ku Tao 5	S^2S^2	NIFTS	Taiwan
74	Kemomno Okinawamishou-2	S^2S^2	NIFTS	Japan (Wild Peach)
75	Noto 6	S^2S^2	NIFTS	Japan (Wild Peach)
76	Zao Xia Lu	S^2S^2	NIFTS	China
77	Khanda	S^2S^2	UC Davis	Pakistan
78	Loimari	S^2S^2	UC Davis	Pakistan
79	Shintanyou	S^2S^2	Okayama	China
80	Juseitou (Hitoe-Shiro)	S^2S^2	Okayama	Japan (Ornamental Peach)
81	Juseitou (Aka-Yae)	S^2S^2	Okayama	Japan (Ornamental Peach)
82	Okinawa 1	S^2S^2	NIFTS	Japan (Wild Peach)
83	Yaseitou 5	S^2S^2	Okayama	Japan (Wild Peach)
84	Yaseitou 6	S^2S^2	Okayama	Japan (Wild Peach)
85	Yaseitou 7	S^2S^2	Okayama	Japan (Wild Peach)
86	Terute Beni	S^2S^2	NIFTS	Japan (Ornamental Peach)
87	Terute Shiro	S^2S^2	NIFTS	Japan (Ornamental Peach)
88	Okayama Yaseitou Tsugawa-3	S^2S^2	NIFTS	Japan (Wild Peach)
89	Zao Hua Lu	S^2S^{2m}	NIFTS	China
90	Chun Lei	S^2S^{2m}	NIFTS	China
91	Rikaku Suimitsu	S^2S^{2m}	Okayama	China
92	Shang Hai Shui Mi Tao	S^2S^{2m}	NIFTS	China
93	Fukusyu	S^2S^{2m}	Okayama	Taiwan
94	Akabana Bantou	S^2S^3	NIFTS	Japan (Ornamental Peach)
95	Shidare Hekitou	S^2S^3	Okayama	China (Ornamental Peach)
96	Yaezaki Bantou	S^2S^3	NIFTS	Japan (Ornamental Peach)
97	Okayama Yaseitou Asahikawa-3	S^2S^4	NIFTS	Japan (Wild Peach)
98	Fei Chang Tao	S^2S^4	Okayama	China
99	Okayama Yaseitou Tsugawa-4	S^2S^4	NIFTS	Japan (Wild Peach)
100	Okayama Yaseitou Tsugawa-5	S^2S^4	NIFTS	Japan (Wild Peach)
101	Kanhitou	$S^{2m}S^{2m}$	NIFTS	Japan (Ornamental Peach)
102	Shen Zhou Shui Mi Tao	$S^{2m}S^{2m}$	NIFTS	China
103	Keihou	$S^{2m}S^{2m}$	Okayama	China
104	Yaseitou 3	$S^{2m}S^{2m}$	Okayama	Japan (Wild Peach)
105	Yaseitou 4	$S^{2m}S^{2m}$	Okayama	Japan (Wild Peach)
106	Okayama Yaseitou Tsugawa-1	$S^{2m}S^{2m}$	NIFTS	Japan (Wild Peach)
107	Okayama Yaseitou Tsugawa-2	$S^{2m}S^{2m}$	NIFTS	Japan (Wild Peach)
108	Kikumomo	S^3S^3	NIFTS	Japan (Ornamental Peach)
109	Sagami Shidare	S^3S^3	NIFTS	Japan (Ornamental Peach)
110	Akita Yaseitou	S^4S^4	NIFTS	Japan (Wild Peach)
111	Chichibu 2	S^4S^4	NIFTS	Japan (Wild Peach)
112	Okayama Yaseitou Koegatouge	S^4S^4	NIFTS	Japan (Wild Peach)
113	Noto 5	S^4S^4	NIFTS	Japan (Wild Peach)
114	Ohatsumomo	S^4S^4	NIFTS	Japan (Wild Peach)
115	Hiley	S^4S^4	UC Davis	Unknown
116	0664. B	S^4S^4	UC Davis	Unknown
117	Stanwick	S^4S^4	UC Davis	Unknown
118	Indian Freestone	S^4S^4	UC Davis	Unknown
119	1469.5 B	S^4S^4	UC Davis	Pakistan
120	1469.7 B	S^4S^4	UC Davis	Pakistan
121	1472.10 B	S^4S^4	UC Davis	Pakistan
122	1473.1 B	S^4S^4	UC Davis	Pakistan
123	1473.10 B	S^4S^4	UC Davis	Pakistan
124	Lutkoo	S^4S^4	UC Davis	Pakistan
125	1485.6 B	S^4S^4	UC Davis	Unknown
126	Dai-Shirobana	S^4S^4	Okayama	Japan (Wild Peach)
127	Jeronimo Balate	S^4S^4	CITA	Spain
128	Jeronimo 2251	S^4S^4	CITA	Spain
129	Zaitani (Anita)	S^4S^4	CITA	United States
130	Baby Gold 9	S^4S^4	CITA	United States

[z] Both *S-RNase* and *SFB* genotypes were determined in this study.

[y] NIFTS: NARO Institute of Fruit Tree Science, UC Davis: University of California, Davis, Okayama: Okayama Research Institute for Agriculture, CITA: Unidad de Fruticultura, CITA de Aragón.

Table 2. DNA sequences of oligonucleotide primers used in this study.

Experiment	Primer name	Sequence (5′-3′)	Reference
S-RNase-based genotyping	Pru-C2	CTATGGCCAAGTAATTATTCAAACC	Tao et al., 1999
	Pru-C4R	GGATGTGGTACGATTGAAGCG	Tao et al., 1999
dCAPS analysis for S^2 and S^{2m}	S2Dra-F	ACAGAAGTTCATATCCACTAATGAA	Tao et al., 2007
	S2Dra-R	CAGCTTTAGCGCATCTATATTCATTT	Tao et al., 2007
S^4-*RNase*-specific amplification	S4-RNase F3	GAAAGCGAATGGAACAAGCA	This work
	S4-RNase R5	AACTGAGTCTTCTTCTTCTG	This work
Insert detection for SFB^1	Pp_SFB1_V1F	TCCACCACCCAAATGTTAGACG	This work
	Pp_SFB1_R1	AACATAGATCTCCTATGCCC	This work
Insert detection for SFB^2 by dCAPS analysis	Pp_SFB2_BSrBI_F	GTTGCTCTCCAATTCGGGTTCCGC	This work
	Pp_SFB2_R3	CTCCTCACAACCATAACATC	This work
Mutation detection for SFB^3	Pp_SFB3_F2	TCCTTCGGGTGATTATTG	This work
	Pp_SFB3_R2N	AATCCGAGCACACCTACG	This work
Insert detection for SFB^4	Pp_SFB4_F5	GTTCCAAACAGAGGCCACAC	This work
	Pp_SFB4_R2	GTGATAGGCTACACCATTGA	This work

and LAS3000-mini (Fuji Film, Tokyo, Japan) for digital images.

Cloning and characterization of the S^3 and S^4 haplotypes

A fosmid library was constructed from the genomic DNA of 'Shidare Hekitou' (S^2S^3) and 'Jeronimo Balate' (S^4S^4) using the CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI, USA) as described previously (Ushjima et al., 2004). The library was screened using the same DIG-dUTP-labeled sweet cherry S^1-*RNase* cDNA probe as that used for the DNA gel blot analysis. Isolated genomic clones that contained the S^3 and S^4 haplotypes were used as templates for the DNA sequencing reaction and PCR analysis to determine the physical distance between *S-RNase* and *SFB* as described previously (Hanada et al., 2009). Deduced amino acid sequences were aligned with other *Prunus S-RNase*s and *SFB*s using the CLUSTALW program version 1.83 provided by GenomeNet (http://www.genome.jp/tools/clustalw/).

Determination of the mutation in SFB

The *SFB* allele-specific primer sets used to detect a mutation in *SFB* were designed to check if a certain cultivar or strain had a mutated *SFB* (Table 2). All PCR reactions contained 1× *ExTaq* buffer, 0.2 mM each of dNTPs, 0.4 μM of each primer, 50 ng template total DNA, and 0.4 U TaKaRa *ExTaq* polymerase (TaKaRa Bio, Shiga, Japan) in a 15-μL reaction volume. PCR amplification was performed using a program with initial denaturation at 94°C for 1 min, 35 cycles of 94°C for 1 min, 56°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR-amplified fragments from SFB^1, SFB^3, and SFB^4 were separated directly in 1% agarose gel electrophoresis and visualized with ethidium bromide under UV light. For SFB^2, 5 μL of the PCR products were digested with 10 U of *Bsr*BI in a 20-μL reaction volume. Digested SFB^2 fragments were separated in 3% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Results

S-RNase genotyping

The PCRs using the Pru-C2/Pru-C4R primer set to amplify the *S-RNase*s of 130 peach cultivars and strains yielded bands with sizes that were different from the expected sizes from S^1- and S^2-*RNase*s. As shown in Figure 1, we detected novel fragments of about 600 bp and 1600 bp that were different in size from the bands for the S^1-, S^2-, and S^{2m}-*RNase*s, which were amplified from several cultivars and strains including 'Shidare Hekitou' and 'Jeronimo Balate'. Because we found that the DNA sequences of the novel PCR bands encoded partial S-RNase sequences, we assigned S^3 and S^4 to the *S-RNase* alleles revealed by these bands. Because we found only homozygotes for S^4-*RNase* in the PCR analyses, we subjected all 130 cultivars and strains to DNA blot analysis using an *S-RNase*-specific probe (Fig. 2). Several strains and cultivars that had heterozygous genotypes, such as S^1S^4 and S^2S^4, were detected; however, no S^3S^4 genotype was found. Because S^4-*RNase* produced longer PCR fragments than the other peach *S-RNase* alleles, PCR amplification of the S^4-*RNase* allele seemed to be competitively prohibited when the S^4-*RNase* allele was present along with other *S-RNase* alleles. Therefore, we occasionally used an S^4-*RNase*-specific primer set to determine the *S-RNase* genotype of the cultivars and strains. *S-RNase* genotyping by both DNA gel blot analyses and PCRs corresponded well when the PCR was performed with both Pru-C2/Pru-C4R and the S^4-*RNase*-specific primer sets. Because S^2-*RNase* and S^{2m}-*RNase* cannot be discriminated by either DNA blot analyses or PCRs with the Pru-C2/Pru-C4R primer set, we used the dCAPS marker to discriminate them. The *S-RNase* genotypes of all analyzed cultivars determined in this study are shown in Table 1.

Cloning and characterization of S locus genes

Genomic DNA libraries of 'Shidare Hekitou' (S^2S^3) and 'Jeronimo Balate' (S^4S^4) were constructed and screened using an *S-RNase* gene-specific probe. Confirmation of the presence of *SFB* and determination of the *S-RNase* allele was performed by PCR analyses. Full-length DNA sequences for the S^3- and S^4-*RNase*s were obtained from the genomic clones that were isolated. Both the S^3- and S^4-*RNase*s seemed to encode an intact S-RNase with no apparent defects. The derived amino acid sequences contained 5 conserved domains, including 2 active sites for RNase catalytic activity, and shared sequence homology with other functional *Prunus* S-RNase within the range of similarities that was observed between other functional S-RNases (Fig. 3). Unlike S^1-, S^2-, and S^{2m}-*RNase*s, no *S-RNase* with high sequence similarity to the S^3- or S^4-*RNase*s was found in the International Nucleotide Sequence Databases (INSD; http://www.insdc.org/) (Tables 3 and 4). Although *SFB* sequences were also present in the genomic clones downstream of the S^3- and S^4-*RNase*s and in reverse transcriptional orientation, as reported in most other functional *Prunus S* haplotypes, both SFB^3 and SFB^4 were mutated (Figs. 4 and 5) and appeared to encode truncated dysfunctional SFBs, as was reported previously for peach SFB^1 and SFB^2 (Fig. 5; Table 5). DNA sequencing of the entire downstream region of SFB^3 extending for about 12 kbp

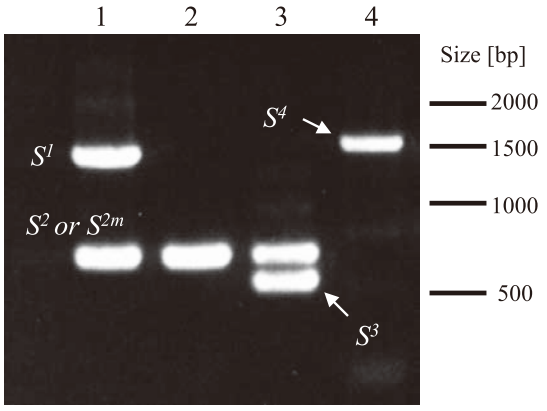

Fig. 1. PCR based S-RNase genotyping of representative peach cultivars using the Pru-C2/Pru-C4R primer set. The *S-RNase* genotypes of 'Shimizuhakuto' and 'Chiyomaru' are known to be S^1S^{2m} and S^2S^2, respectively. The unidentified bands in 'Shidare Hekitou' and 'Jeronimo Balate' were named S^3 and S^4, respectively. Lane 1, 'Shimizuhakuto' (S^1S^{2m}); lane 2, 'Chiyomaru' (S^2S^2); lane 3, 'Shidare Hekitou' (S^2S^3); and lane 4, 'Jeronimo Balate' (S^4S^4).

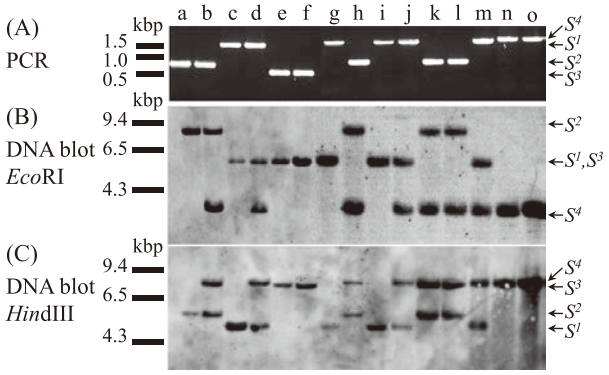

Fig. 2. S-RNase genotyping by PCR and DNA gel blot analyses. (A) PCR genotyping using the S-RNase-specific Pru-C2/Pru-C4R primer set. (B) S-RNase genotyping by DNA blot analysis with *Eco*RI digestion. (C) S-RNase genotyping by DNA blot analysis with *Hin*dIII digestion. Lanes a, 'Yaseitou 4'; b, 'Fei Chang Tao'; c, 'Nagano Yaseitou-Wase'; d, 'Nagano Yaseitou-Bansei'; e, 'Kikumomo'; f, 'Sagami Shidare'; g, 'Okayama Yaseitou Asahikawa-2'; h, 'Okayama Yaseitou Asahikawa-3'; i, 'Okayama Yaseitou Kamogawa-1'; j, 'Okayama Yaseitou Kamogawa-2'; k, 'Okayama Yaseitou Tsugawa-4'; l, 'Okayama Yaseitou Tsugawa-5'; m, 'Okayama Yaseitou Asahikawa-1'; n, 'Dai-Shirobana'; and o, 'Okayama Yaseitou Koegatouge'.

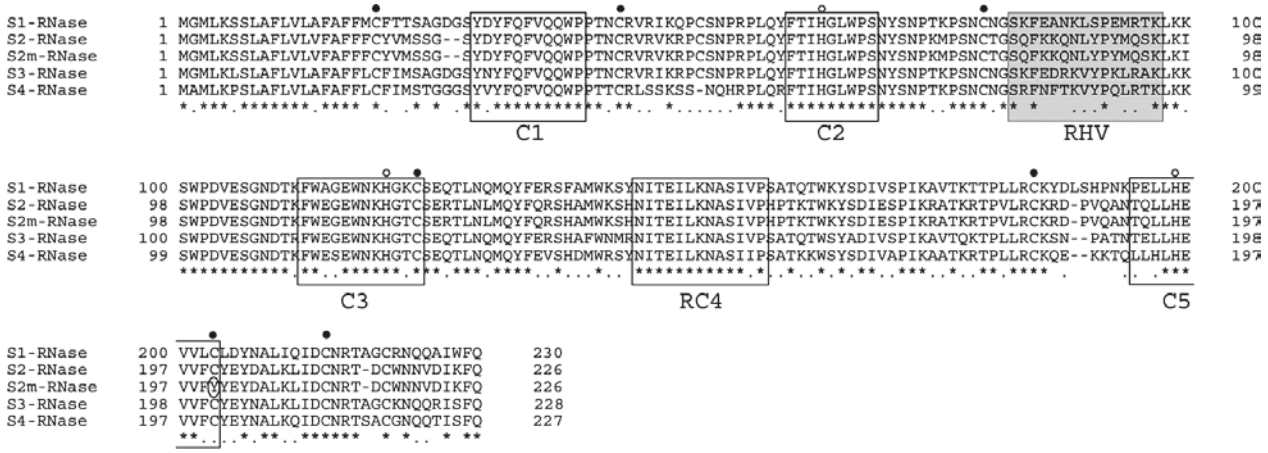

Fig. 3. Alignment of the deduced amino acid sequences of peach S-RNases. The sequences of the S^1-, S^2-, and S^{2m}-RNases were reported previously (Tao et al., 2007). Five conserved domains of rosaceous S-RNase (C1, C2, C3, RC4, and C5) are indicated in open boxes. The rosaceous hypervariable region (RHV) is indicated in a gray box. Conserved histidine residues essential for RNase catalytic activity are indicated by open circles, conserved cysteine residues are marked with closed circles, respectively above the alignment. The tyrosine residue in S^{2m}-*RNase*, which is thought to be mutated from the conserved cysteine residue, is circled. The INSD accession numbers of S^1-*RNase*, S^2-*RNase*, S^{2m}-*RNase*, S^3-*RNase*, and S^4-*RNase* are AB252415, AB252317, AB597186, AB537563, and AB537565, respectively.

Table 3. Derived amino acid sequence identities (%) of *Prunus* SFB (upper half) and S-RNases (lower half).

	P. avium		*P. armeniaca*		*P. dulcis*		*P. mume*		*P. salicina*		*P. cerasus*	*P. speciosa*	*P. persica*				
	PavS^{2}	PavS^{13}	ParS^{1}	ParS^{2}	PdS^{a}	PdS^{k}	PmS^{1}	PmS^{7}	PsS^{a}	PsS^{e}	PcS^{26}	PspS^{1}	PpS^{1}	PpS^{2}	PpS^{2m}	PpS^{3}	PpS^{4}
PavS^{2}	—	79.8	85.6	77.6	66.1	79.7	81.6	79.5	76.6	76.3	79.7	78.7	79.2	77.6	—	80.9	82.4
PavS^{13}	76.4	—	78.5	77.1	65.2	78.7	80.2	76.3	76.3	78.1	80.3	79.0	78.4	77.4	—	84.3	84.4
ParS^{1}	83.2	77.3	—	79.5	66.6	80.2	80.8	77.9	78.2	76.8	78.7	77.9	80.3	78.7	—	81.5	80.3
ParS^{2}	74.8	75.1	75.7	—	67.2	80.8	81.6	79.7	76.8	80.2	77.1	77.1	80.5	77.3	—	79.6	76.5
PdS^{a}	50.4	54.2	54.6	49.1	—	69.1	68.8	68.7	66.2	66.1	68.3	66.8	68.5	67.3	—	70.4	67.6
PdS^{k}	81.4	71.1	75.5	72.6	51.1	—	80.6	80.6	80.0	77.3	80.0	81.1	99.2	80.5	—	81.5	79.2
PmS^{1}	81.3	71.0	76.3	68.3	54.0	74.1	—	80.3	78.7	79.2	80.5	79.5	80.0	79.5	—	81.2	81.6
PmS^{7}	71.2	74.2	70.4	67.7	50.0	67.7	72.8	—	75.8	77.6	78.9	77.7	80.3	77.4	—	81.8	78.2
PsS^{a}	73.9	69.3	74.8	69.9	52.7	71.7	66.5	64.2	—	74.9	78.4	84.6	79.5	97.9	—	77.2	77.1
PsS^{e}	81.9	73.8	82.5	76.5	52.9	76.1	77.7	73.0	70.8	—	74.4	76.0	77.1	75.7	—	77.2	76.3
PcS^{26}	77.0	73.8	74.8	70.4	49.1	72.1	74.1	77.9	66.8	77.4	—	78.9	79.7	78.7	—	80.9	79.7
PspS^{1}	78.8	75.1	77.9	73.0	50.9	76.1	69.2	70.4	77.9	77.0	73.9	—	80.5	85.6	—	79.0	79.3
PpS^{1}	81.5	71.1	75.5	72.6	51.1	100.0	74.1	67.7	71.7	76.1	72.1	76.1	—	80.0	—	81.2	78.7
PpS^{2}	73.9	68.4	74.3	69.5	53.1	71.7	66.5	63.7	97.8	70.8	66.8	78.3	71.7	—	—	78.7	78.2
PpS^{2m}	73.5	68.0	73.8	69.1	52.7	71.2	66.1	63.3	97.3	70.4	66.4	77.9	71.2	99.6	—	—	—
PpS^{3}	83.2	77.3	78.9	74.8	52.9	81.1	75.0	72.1	72.6	80.7	74.8	83.6	81.1	72.1	71.7	—	83.3
PpS^{4}	76.4	81.8	75.1	70.7	53.8	70.7	70.5	80.4	65.3	75.1	84.4	74.7	70.7	66.2	65.8	75.6	—

For peach SFB^{1}, SFB^{2} (Tao et al., 2007), and SFB^{4}, the putative original sequences that were derived from original *SFB* sequences reverted by removing the inserted sequence were used to calculate identities. Pav, *P. avium*; Par, *P. armeniaca*; Pd, *P. dulcis*; Pm, *P. mume*; Ps, *P. salicina*; Pc, *P. cerasus*; Psp, *P. speciosa*; and Pp, *P. persica*. The sequences used are as follows; Pav-S^{2}-RNase (AJ298311), Pav-S^{13}-RNase (DQ385842), Par-S^{1}-RNase (AY587561), Par-S^{2}-RNase (AY587562), Pd-S^{a}-RNase (AB026836), Pd-S^{k}-RNase (AB252409), Pm-S^{1}-RNase (AB101438), Pm-S^{7}-RNase (AB101439), Ps-S^{a}-RNase (AB252411), Ps-S^{e}-RNase (AB280793), Pc-S^{26}-RNase (EU035975), Psp-S^{1}-RNase (GU968644), Pp-S^{1}-RNase (AB252415), Pp-S^{2}-RNase (AB252417), Pp-S^{2m}-RNase (AB597186), Pp-S^{3}-RNase (AB537563), Pp-S^{4}-RNase (AB537565), Pav-SFB^{2} (AB111519), Pav-SFB^{13} (DQ385844), Par-SFB^{1} (AY587563), Par-SFB^{2} (AY587562), Pd-SFB^{a} (AB092966), Pd-SFB^{k} (AB252408), Pm-SFB^{1} (AB101440), Pm-SFB^{7} (AB101441), Ps-SFB^{a} (AB252410), Ps-SFB^{e} (AB280794), Pc-SFB^{26} (EU035977), Psp-SFB^{1} (HM347508), Pp-SFB^{1} (AB252414), Pp-SFB^{2} (AB252416), Pp-SFB^{3} (AB537564), and Pp-SFB^{4} (AB537566).

Table 4. DNA and derived amino acid length of peach *S-RNases*.

Species	Allele	Accession^{z}	Reference	Length in the genome (bp)^{y}	CDS (bp)^{x}	No. amino acid	Note
P. persica	*S^{1}*	AB252415	Tao et al., 2007	1884	693	230	
	S^{2}	AB252417	Tao et al., 2007	1343	681	226	
	S^{2m}	AB597186	Tao et al., 2007	1343	681	226	A single amino acid substitution in the C5 region of S^{2}-RNase
	S^{3}	AB537563	This work	1197	687	228	
	S^{4}	AB537565	This work	2150	678	225	
P. dulcis	*S^{k}*	AB252409	Tao et al., 2007	1888	693	230	Encoding the same amino acid sequence as *P. persica S^{1}-RNase*
P. salicina	*S^{a}*	AB252411	Tao et al., 2007	1277	681	226	Encoding the same amino acid sequence as *P. persica S^{2}-RNase*

^{z} International Nucleotide Sequence Databases (INSD; http://www.insdc.org/) accession number.
^{y} Start codon to stop codon with introns.
^{x} No. of nucleotide from the start codon to stop codon.

revealed the absence of a sequence homologous to *SFB*. There was a 4946 bp insertion (4244 bp insertion flanked by 351 bp direct repeats) in the middle of *SFB^{4}*. The original *SFB^{4}* sequence can be obtained by removing the inserted sequence, and the reverted sequence encodes a typical SFB with the F-box motif at the N-terminus (Figs. 4 and 5). The predicted original SFB^{4} shared 70–80% amino acid identity with other SFBs. Peach SFB^{3} and SFB^{4} showed the highest amino acid sequence homology to *P. avium* SFB^{13}, with 84.3% and 84.4% amino acid identity, respectively (Table 3). Physical distances between *S-RNase* and *SFB* in *S^{3}* and *S^{4}* haplotypes of peach were 12 kb and 4.3 kb, respectively (Fig. 5).

Mutation in SFB

The PCR primer sets that were used to detect mutations in peach *SFB*s were designed to test if the *S* haplotypes in all the peach cultivars and strains used in this study were mutated. To detect the presence or absence of the insertion in *SFB^{1}*, we designed a primer set that amplified the *SFB^{1}* region that contained inserted sequences. If the insertion was present, the amplified products would

Fig. 4. Alignments of the DNA sequences and derived amino acid sequences of peach *SFB*[3], *SFB*[4], and *P. avium SFB*[13]. (A) DNA sequence alignment of *P. avium SFB*[13] (PavSFB13), *P. persica SFB*[3] (PpSFB3), *SFB*[4] (PpSFB4) with the 6 bp inserted sequence that contains a stop codon, and *SFB*[4] reverted by removing the inserted sequence (PpSFB4-4946). The gray box indicates the 6 bp front position of the inserted sequence in preach *SFB*[4]. (B) Amino acid sequence alignment of deduced proteins from *P. avium SFB*[13] (PavSFB13), *P. persica SFB*[3] (PpSFB3), *P. persica SFB*[4] (PpSFB4), and *P. persica SFB*[4] reverted by removing the inserted sequence (PpSFB4-4946). The dotted box indicates the F-box motif. Two of each variable region (V1, V2) and hypervariable region (HVa and HVb) are indicated by open and gray boxes, respectively. The INSD accession numbers of *P. avium SFB*[13], *P. persica SFB*[3], and *P. persica SFB*[4] are DQ385844, AB537564, and AB537566, respectively.

be longer than the products from the original intact *SFB*. We used almond *SFB*^{k}, an original intact functional type *SFB* of *SFB*^{1}, as a reference. As shown in Figure 6, *SFB*^{1} from all peach cultivars and strains used in this study yielded longer products than almond *SFB*^{k}, indicating that there was no original functional *SFB*^{1} in any of the peach cultivars and stains tested. Because the inserted sequence to *SFB*^{2} was only 5 bp long, it was difficult to distinguish the presence of the insertion by length polymorphism. We therefore developed a dCAPS marker to distinguish the original *SFB* and the mutated *SFB*^{2} alleles following the strategy used by Ikeda et al. (2004) to develop dCAPS markers for sweet cherry *SFB*$^{4'}$. After *Bsr*BI digestion, the PCR product from mutated *SFB*^{2} should be shorter than the product from Japanese plum *SFB*^{a}, the original functional type *SFB*^{2} with no insertion. We found that *SFB*^{2} in all the peach cultivars and strains used in this study were mutated *SFB*s with 5 bp insertions. A reverse primer for the amplification of *SFB*^{3} and a forward primer for *SFB*^{4} were designed from the sequences that were absent in the original functional alleles. Therefore, only mutated *SFB* alleles were amplified by PCR. All *SFB*^{3} and *SFB*^{4} in the peach cultivars and strains used in this study appeared to be mutated *SFB*s (Fig. 6).

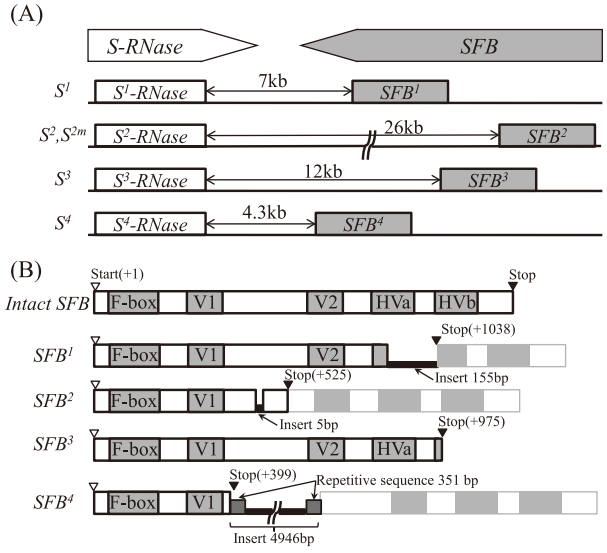

Fig. 5. Schematic diagrams illustrating the organization of *S-RNase* and *SFB* in the peach *S* locus region and the structure of peach *SFB*s. (A) Schematic diagram of the organization of *S-RNase* and *SFB* in the genomic sequence. Open and gray boxes indicate the *S-RNase* and *SFB* coding regions, respectively. The transcriptional orientations of *S-RNase* and *SFB* are in opposite directions relative to one another. (B) Schematic diagram of truncated peach SFBs. Gray boxes indicate the conserved structures (F-box, V1, V2, HVa, and HVb). Light gray boxes indicate the truncated region caused by the insertion and frameshift. The inserted sequence and repetitive sequence are indicated by black and dark gray boxes, respectively.

Discussion

This study showed that 2 novel SC PPM *S* haplotypes were present in peach in addition to the 3 SC PPM *S* haplotypes, *S*^{1}, *S*^{2}, and *S*^{2m}, which were identified previously. Our preliminary survey of the *S* haplotypes of over 300 diverse peach cultivars and lines indicated that no more novel *S* haplotypes existed (Hanada and Tao, unpublished data), although some mutated versions of the existing *S* haplotypes may exist, as seen in the case in *S*^{2m} and *S*^{2}. The small number of *S* haplotypes may indicate that peach experienced a population bottleneck and/or positive selection on the mutated SC *S* haplotypes. Because peach is a domesticated plant, the domestication process may have affected the population bottleneck and/or positive selection on self-compatibility. However, most of the peach-related wild species in the *Prunus* subgenus *Amygdalus*, such as *P. mira*, *P. davidiana*, and *P. kasuensis*, are predominantly SC (Tao, Hanada, Akagi and Gradziel, unpublished data), which makes this inference complicated. It is unclear whether the population bottleneck and/or positive selection occurred upon peach

Table 5. Length of peach *SFB* and ther inserted sequence.

Species	Allele	Accession[z]	Reference	Inserted sequence (bp)	CDS (bp)[y]	No. amino acid	Note
P. persica	*SFB*^{1}	AB252414	Tao et al., 2007	155	1098	365	Mutant of *P. dulcis SFB*^{k} with a 155-bp insertion.
	SFB^1 (reverted[x])			—	1128	375	
	SFB^2	AB252416	Tao et al., 2007	5	525	174	Mutant of *P. salicina SFB*^{a} with a 5-bp insertion.
	SFB^2 (reverted)			—	1131	376	
	SFB^3	AB537564	This work	Unknown[w]	975	324	Stop codon appeared at the positon 975-bp from the start codon
	SFB^4	AB537566	This work	4946	399	132	A 4946-bp insertion mutation
	SFB^4 (reverted)			—	1131	376	
P. dulcis	*SFB*^{k}	AB252408	Tao et al., 2007		1128	375	
P. salicina	*SFB*^{a}	AB252410	Tao et al., 2007		1131	376	

[z] International Nucleotide Sequence Databases (INSD; http://www.insdc.org/) accession number.
[y] No. of nucleotide from the start codon to stop codon.
[x] Reverted original allele by removing the inserted sequence.
[w] Neither the downstream sequence or the original stop codon of *SFB*^{3} was found in the 12-kb downstream region from the stop codon of *SFB*^{3} to the stop codon of *S*^{3}*-RNase*.

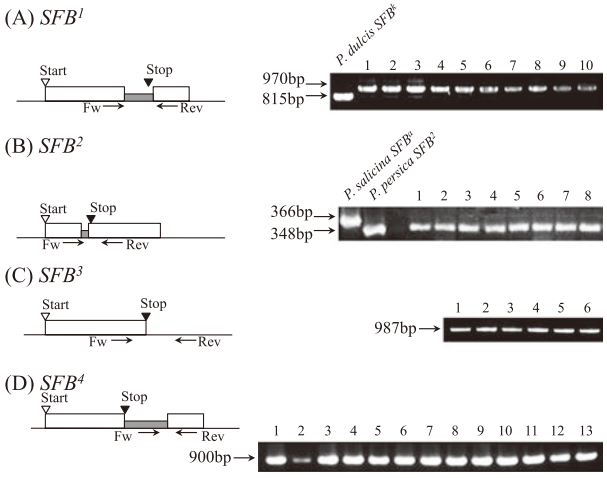

Fig. 6. Detection of mutation in the coding regions of peach *SFB*s by PCR analysis. A specific primer pair for each *SFB* allele was designed to detect mutation. Open boxes indicate intact coding regions. Start and stop codon positions are indicated by open and closed triangles, respectively. Arrows indicate the positions of the forward (Fw) and reverse (Rev) primers. (A) PCR amplification to detect the insertion in *SFB*^{1}. Almond *SFB*^{k}, a wild type of *SFB*^{1}, was used as a control. Lane 1, 'Jing Hong'; lane 2, 'Terute Suimitsu'; lane 3, 'Nagano Yaseitou-Wase'; lane 4, 'Nagano Yaseitou-Bansei'; lane 5, 'Noto 2'; lane 6, 'Noto 3'; lane 7, 'Noto 8'; lane 8, 'Yaezaki Bantou O.P. No. 1'; lane 9, 'Okayama Yaseitou Kamogawa-1', and lane 10, 'Okayama Yaseitou Asahikawa-1'. (B) The dCAPS marker to detect inserted sequence in *SFB*^{2}. *P. salicina SFB*^{a}, a wild type of *SFB*^{2}, was used as the control. Amplified fragment from *P. persica SFB*^{2} was detected as different sizes after *Bsr*BI digestion. Lane 1, 'Akashidare'; lane 2, 'Akabana Bantou'; lane 3, 'Akahayazaki'; lane 4, 'Amami Yaseitou-1'; lane 5, 'Amami Yaseitou-2'; lane 6, 'Da Tao'; lane 7, 'Okinawa 1', and lane 8, 'Kimumu Nakamineyuumei'. (C) PCR amplification to detect mutation in *SFB*^{3}. Lane 1, 'Kikumomo'; lane 2, 'Sagami Shidare'; lane 3, 'Akabana Bantou'; lane 4, 'Yaezaki Bantou O.P. No. 1'; lane 5, 'Yaezaki Bantou', and lane 6, 'Shidare Hekitou'. (D) PCR amplification to detect insertion in *SFB*^{4}. Lane 1, 'Okayama Yaseitou Asahikawa-1'; lane 2, 'Okayama Yaseitou Asahikawa-3'; lane 3, 'Okayama Yaseitou Tsugawa-4'; lane 4, 'Okayama Yaseitou Tsugawa-5'; lane 5, 'Okayama Yaseitou Kamogawa-2'; lane 6, 'Chichibu 2'; lane 7, 'Noto 5'; lane 8, 'Okayama Yaseitou Koegatouge'; lane 9, 'Fei Chang Tao'; lane 10, 'Ohatsumomo'; lane 11, 'Akita Yaseitou'; lane 12, 'Nagano Yaseitou-Bansei'; and lane 13, 'Dai-Shirobana'.

speciation from its progenitor species or before peach speciation. Population genetic approaches and investigation of the *S* locus and *S* haplotype in peach-related *Amygdalus* species could give important clues to address the question.

In *Prunus*, dysfunction of either the pistil *S* determinant *S-RNase* or the pollen *S* determinant *SFB* confers self-compatibility. Thus, if evolutionary constraints or selection could be disregarded, the rate of mutation needed to confer self-compatibility would be equal for both the pistil and pollen parts in *Prunus*. Although the coding sequence of *SFB* is 1.5 times longer than that for *S-RNase*, the *S-RNase* sequence from the initiation codon to the termination codon is longer than the *SFB* sequence because of the presence of introns in the *S-RNase* sequence. Considering that the causal factor of self-compatibility in peach is a mutation in pollen *S* for all the *S* haplotypes found, the mutation in pollen *S* may have been preferentially selected. As we proposed previously (Tao et al., 2007), the mutation in pollen *S* may have been selected preferentially compared with the pistil part mutants under selection pressure for SC because the pollen genotype determines the self-incompatible phenotype of pollen in the GSI system. Namely, a mutation in *SFB* that occurs in a single pollen grain could confer self-compatibility to the original pollen grain in which the mutation first occurs. Then the SC phenotype would be transmitted to the second generation, in which the pollen grain would participate in fertilization either after self- or cross-pollination, while a mutation in *S-RNase* in a single pollen grain would be unable to confer self-compatibility to the pollen and would be only transmittable to the progeny after cross-pollination because mutations in *S-RNase* would have no effect on the SC/SI phenotype of the pollen grain. We therefore suppose that the mutation in pollen *S* would be preferentially selected under selection pressure for SC in the GSI system. If our hypothesis is correct, peach has experienced positive selection for SC in its evolutionary path.

On the practical side, this study could give us important indications of how we can breed SC cultivars in *Prunus* fruit tree species, in which one of the major breeding goals is SC. Current SC breeding in *Prunus* is exclusively accomplished by cross breeding using existing SC strains as a parent. For example, almost all SC sweet cherry (*P. avium*) cultivars recently released are offspring of JI2420, which is a SC strain produced by X-ray irradiation breeding (Lewis, 1949; Ushijima et al., 2004). SC 'NK14' Japanese apricot (*P. mume*) is from crosses between self-incompatible 'Nanko' and SC 'Kensaki', a naturally occurring PPM SC cultivar. However, considering the astronomical number of pollen grains present in a single flower and that a mutation in *SFB* in a single pollen grain could confer self-compatibility to the pollen grain itself, we should be able to more effectively utilize spontaneous or artificial mutation in *SFB* for SC breeding, as the SC PPM *S*$^{4'}$ haplotype was artificially produced in sweet cherry (Lewis, 1949).

Acknowledgements

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